Mulberry juice freeze-dried powder attenuates the disease severity by the maintaining of colon mucosa in mice with DSS-induced acute colitis

Abstract: This study aimed to evaluate the microbial compositions and gene expression related to inflammation in dextran sodium sulfate (DSS)-induced acute colitis and the effect of mulberry supplementation. Male BALB/c mice received a diet supplemented with mulberry juice freeze-dried powder (MFP) or not for 3 weeks. After 3 weeks, the mice received water containing 5% (w/v) DSS or not for 1 week. The disease activity index score in mice fed MFP was significantly decreased. A significant decrease in *Bifidobacterium* spp. and the *Clostridium perfringens* subgroup was observed in mice not fed MFP. The number of goblet cell and NLRP6 expression were observed in mice fed a diet supplemented with MFP compared with mice not fed MFP. These results may indicate that mulberry mitigates DSS-induced acute colitis by a changing the gut microbial flora and by improving mucosal conditions.

Keywords: Mulberry, DSS-induced acute colitis, Goblet cell, NLRP6 Inflammasome, Microbiota.

Introduction

Cases of inflammatory bowel disease (IBD) are increasing worldwide [1]. The most common forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD). The symptoms of IBD include repeated abdominal pain, bloody feces, diarrhea, and fever, which together greatly reduce the patients' quality of life [2]. Currently, the underlying mechanisms leading to UC and IBD remain unclear while environmental factors have been reported to be involved in their disease etiology. The disease pathogenesis widely accepted consists of the disruption of the epithelial barrier, triggering an invasion of bacteria into the mucosal layer, which then activates the inflammatory immune response. The model is useful to study the contribution of innate immune systems in intestinal inflammation and presumably due to the toxic effects of dextran sodium sulfate (DSS) on epithelial barrier dysfunction. UC development is strongly correlated with oxidative stress by reactive oxygen species (ROS) [3]. The inflammatory process is accompanied by immunological cell infiltration and the unregulated production of proinflammatory cytokines and ROS. High levels of ROS are generated by immune cells such as infiltrated active macrophages in the colon, resulting in responses that prolong inflammation. Increased levels of proinflammatory cytokines are detected in patients with IBD.

Several families of innate receptors are involved in the recognition of microbe-associated molecular patterns, such as Toll-like receptors and nucleotide-binding oligomerization domain protein-like receptors (NLRs) [4]. Inflammasomes are innate sensors in which apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) connects the sensing of microbial molecular patterns or cellular stress by NLRs to Caspase-1 (Casp-1)-mediated maturation of interleukin (IL-)-1β and IL-18 [5]. The results in the study using Casp-1 knockout mice have

strongly suggested that Casp-1 plays a key role in DSS-induced colitis [6]. The NLRP3 inflammasome plays a key role in inflammation because disease-associated NLRP3 mutations enhance Casp-1 activation and IL-1β production [7]. Furthermore, a decreased NLRP3 level and decreased IL-1β secretion were recently reported to be linked with increased susceptibility to CD in humans [8]. On the other hand, ASC-dependent inflammasomes, such as NLRP6, were identified as a key immune axis regulating the intestinal microbiota [9]. The NLRP6 inflammasome has been shown to play critical roles in defense against infection, autoinflammation, and tumorigenesis. As NLRP6 is highly expressed in epithelial cells, especially goblet cells, this inflammasome has been considered an essential factor for mucosal self-renewal, cell proliferation, and regulation of intestinal flora through mucus secretion and anti-microbial peptide production from goblet cells [10] and the epithelium [11].

The composition of the intestinal flora was associated with the pathogenesis of UC and CD. The gut microbiota influences the homeostasis of the intestinal mucosa via its barrier function [12]. Mucosal barrier integrity is essential to blocking the access of microorganisms to underlying tissues. Studies in a rodent model have linked tissue damage and the disruption of the epithelial barrier in the gut to cytokine imbalances [13]. UC is generally recognized as an immune-mediated disorder resulting from the abnormal interaction between intestinal microbiota and mucosal immune cells. Differential compositions of intestinal microbiota have been reported in patients with IBD [14]. The strictly anaerobic bacteria, *Bacteroides* spp. plays a vital role in the induction of colitis [15-18]. Bacterial content analysis shows that *Bacteroides* spp. is highly represented in patients with UC but are present in lower amounts in healthy adults [19]. Two bacteria species, *Bifidobacterium* spp. and the *Clostridium perfringens* group, are known as beneficial factors to UC [20-22].

Current therapies for patients with IBD have limited efficacy and present potentially serious side-effects on long-term use [23]. Hence, along with the development of new drugs, supplements might represent, in combination, an important approach. Recently, natural plants containing polyphenols have attracted wide attention because polyphenols have anti-inflammatory properties. For example, grape pomace extracts depress colonic inflammation by preventing the expression of proinflammatory genes and regulating oxidative stress in colitis [24].

We have focused on the mulberry, already used in traditional medicine, rich in beneficial ingredients such as polyphenols, especially anthocyanin [25-27]. The aim of this study was to determine the inhibitory effect of mulberry against UC and understand its mode of action in DSS-induced colitis in the mouse model.

Materials and Methods

Diet preparation and reagents

Mulberry used in this study was harvested in the Shiraishi Island of Okayama prefecture. The contents of anthocyanins, as major polyphenols in this fruit, were 80 mg/g of cyanidin-3-glucoside and 24 mg/g of cyanidin-3-rutinoside. Mulberry juice freeze-dried powder (MFP) from the 5 x concentrated mulberry fruit juice kindly given by Okayamaken Seikabutsu Hanbai Co. Ltd (Okayama, Japan) was prepared in the freeze dryer (TD-81TA, Tokyo Rikakikai *Co., Ltd.*, Tokyo, Japan). The anthocyanin content was 7 mg/g MFP. MFP was stored at -20˚C until use. MFP-containing diet was prepared as a mix with the normal diet (AIN93G: Oriental Yeast *Co., Ltd.*, Tokyo, Japan). DSS 5000 was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan).

Animals and experimental design

BALB/c mice (male, 7 weeks old) were purchased from Charles River Laboratories Japan, Inc (Osaka, Japan). The animals were housed at a constant temperature (22 ± 1 ˚C) with a 12-h dark/light cycle and had access to tap water and food. All procedures were approved by the Animal Care and Use Committee, Okayama University (OKU-2017096) and were conducted in accordance with the Policy on the Care and Use of the Laboratory Animals, Okayama University.

The experimental periods were 28 days in total. In the first 3 weeks, mice (7 weeks old) were randomly divided into 6 groups (n = 3) and were maintained on different diets or drinking water, as shown in Table 1. Briefly, the diet with (Group 1 to 4) or without (Group 5 and 6) MFP (final anthocyanin concentration: 50 mg/kg) was supplied daily for the first 3 weeks. Then, the mice in Groups 1, 3, and 5 were supplied drinking water containing 5% (w/v) DSS for 1 week; and body weight and fecal condition in mice were monitored daily. The mice in Groups 2, 4, and 6 were supplied with normal water. At

the same time, the mice (in Groups 1 and 2) received a fourth week supply of the diet with MFP. At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected, and lengths measured. The disease activity index (DAI) scores were calculated according to the fecal condition and body weight loss (Table 2) as described elsewhere [28]. Then, the colon was divided into three parts after tissue collection. The proximal part of colon was immediately frozen at -80˚C for gene expression analysis. Both middle and distal colon were fixed with 10% formaldehyde for histopathological observation.

Histopathological examination

The middle and distal regions were embedded in paraffin, sectioned at 5-μm thickness, and de-paraffinized. Sectioned specimens were stained using a hematoxylin eosin (HE) solution. HE specimens were observed under the light microscope (Olympus FSX100, Olympus, Tokyo, Japan) to evaluate the tissue damage and to count the number of goblet cells (magnification, x200 and x400). The microscopic score was calculated according to the scoring system as described previously (Table 3) [29]. HE specimens (3 specimens/each region/mouse) were made, and then the number of goblet cells were counted in three fields of each specimen under the light microscope (magnification, x 400).

Gene expression analysis

Total RNAs were extracted from colonic tissues in each mouse using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. The concentration of RNA was quantified using SmartSpec plus Spectrophotometer (Bio-Rad Laboratories Inc., Tokyo, Japan). One microgram of total RNA was subjected to reverse transcription with oligo (dT_{18}) primers using the First Strand cDNA synthesis

kit (Takara Biotechnology, Shiga, Japan) according to manufacturer's instructions. All the cDNA preparations were stored at -20˚C until further use.

The quantitative real-time polymerase chain reaction (qRT-PCR) was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX). Expression values were normalized to GAPDH in the same sample and then normalized to the control. The sequences of the primer pairs used for qRT-PCR amplification are listed in Table 4. Samples were heated at 95˚C for 5 min and then subjected to 40 cycles of denaturation at 95˚C for 5 sec and annealing/elongation for 10 sec at annealing/elongation temperatures described in Table 4. The amplifications were performed on three independent samples, with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta CT}$ method.

Assessment of bacterial contents using qPCR

Fecal DNA samples from mice in all the 6 groups were extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. qPCR amplification and detection were performed in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies).

To determine of the number of *Bacteroides* spp., *Bifidobacterium* spp., and *C. perfringens* subgroup present in each sample, fluorescent signals detected from serial dilutions in the linear range of the assay were averaged and compared to a standard curve generated with standard plasmid DNA in the same experiment. The primer pairs and qPCR conditions used were those described by Rinttilä *et al.* [30]. The resulting levels of total bacteria were assessed using a 16S specific primer pair (357f: 5'-CTCCTACGGGAGGCAGCAG-3' and 517r: (5'-ATTACCGCGGCTGC TGG-3').

Samples were heated at 95˚C for 5 min and then subjected to 35 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 20 sec. The 16S rRNA sequences from each bacterial strain [*Bacteroides fragilis* (JCM 11019^{T}), *Bifidobacterium longum* (JCM 1217^{T}), *C. perfringens* (JCM 1290 ^{T}), and *Escherichia coli* (JCM 1649^{T})] were cloned. The plasmid inserted with the 16S rRNA sequence from each bacterial strain was used to define the standard curve.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SE) or the mean ± standard deviation (SD) of three independent experiments. Data were statistically evaluated by one-way analysis of variance followed by Tukey's HSD using IBM SPSS Statistics software. Values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant.

Results

Body weight gain, colon length, and disease activity

Prior to DSS administration, body weight did not differ among mice groups (data not shown). The body weight after DSS administration is shown in Fig. 1A. The body weight in mice of the DSS treatment groups (Groups 1, 3, and 5) was decreased after DSS administration. In Group 5 mice compared with Group 6 mice, the body weight gain was decreased by about 25%. The reduction in body weight in Group 1 mice was delayed by 2 days compared with that of Group 5 mice (weight decrease in Group 3 mice occurred in the time intermediately). The colon length was significantly shortened in the DSS administration group without MFP (Group 5) (Fig. 1B). The DAI score increased gradually over the last 3 days in the DSS administration group (Fig. 1C). However, disease signs appeared later in the MFP-administered group (Group 1). The DAI score in Group 1 mice was significantly lower than those in Group 3 and Group 5 mice. Thus, MFP significantly attenuated the clinical symptoms of colitis (Fig. 1C).

Histopathology

Both middle and distal colon regions were observed for histopathological examination. In mice administered DSS, the colons showed a destruction of the epithelial layer and the infiltration of inflammatory cells, such as neutrophils, extending through the mucosa and sub-mucosa (Fig. 2A). MFP administration attenuated the extent and severity of lesions and improved the epithelium architecture. The histological scores were calculated according to these observations, with a lower score in Group 1 mice than in Group 5 mice (Fig. 2B). In Group 1, MFP administration allowed only a moderate inflammatory infiltration, indicating an important reduction of colon damage. The number of goblet cells in Group 1 epithelia was significantly higher than those in Groups 3 or 5 (Fig. 2C).

Gene expression analysis

The expression of proinflammatory cytokines and NLRP3 was down-regulated by MFP administration (Fig. 3). The mRNA expression levels of IL-1β, tumor necrosis factor (TNF-)-α, and NLRP3 in Group 3 were higher than those in Group 1. Both NLRP6 and ASC in Group 1 seemed to be expressed at higher levels than those in Groups 3 or 5, but the differences were not statistically significant (G1 vs G3: $p = 0.347$; G1 vs G5: $p = 0.163$).

Bacterial content analysis

qPCR analyses were performed to quantity *Bacteroides spp.*, *Bifidobacterium spp.*, and *C. perfringens* subgroup in fecal samples (Fig. 4). The total bacterial population count was $10^{9\text{-}10}$ copies/g in control fecal samples, and a significant decrease was observed in the total bacterial contents in Group 3 mice fecal samples. The decrease of the *Bifidobacterium* spp. copy number in both Group 3 and 5 mice was significant when compared with Group 1 mice ($p < 0.05$). A significant increase was observed for the *Bacteroides* spp. population in group 5 compared with Group 3 mice ($p < 0.05$). The *C. perfringens* subgroup population in Group 1 mice was much higher than that in both Groups 3 and 5 ($p < 0.05$).

Discussion

IBD is a chronic pathology resulting from uncontrolled inflammation that ultimately leads to mucosal disruption and ulceration. Following epithelial barrier dysfunction, bacteria invade the mucosal layer, resulting in the activation of immune cells. In particular, macrophages play a key role in this disease pathogenesis because they release proinflammatory cytokines and ROS [31]. Indeed, the infiltration of a large number of macrophages is observed in the mucosa during active colitis [32]. ROS is an important factor in the mechanism of IBD pathogenesis. In the context of limited therapy options for patients with IBD, biological and clinical benefits, such as inflammation modulation, was recently reported for fruits, such as berries, containing anthocyanin. Anthocyanins are non-toxic water-soluble pigments with great anti-oxidative capacity. Anthocyanins from blueberry and grape have proven to mitigate IBD symptoms [33]. In this study, we have focused on the mulberry fruit richer in anthocyanin content than other berries. Anthocyanins are major functional polyphenols in this fruit, probably explaining their use in China and Japan in traditional medicines against various diseases [34-39].

The aim of this study was to determine the effect of mulberry against UC and on the mouse intestinal microbiota in DSS-induced colitis. Our results indicate that MFP reduces the major symptoms of DSS-induced acute colitis, including body weight loss, colon shortening, and colonic inflammation (Fig. 1). Feeding the mice, a MFP mixed diet modified the colonic microenvironment, which may play an important role in reducing DSS-induced colonic injury and inflammation and/or maintaining the gut barrier, as evidenced by the reduced DAI score and histological damage, and the increased goblet cell number. Moreover, few infiltrated immune cells were observed suggesting that MFP can protect the mucosal barrier and suppress the activation of

macrophages. Thus, the oral administration of MFP significantly mitigates the disease severity in DSS-induced acute colitis, suggesting that a daily supplement of MFP has health benefits.

The NLRP3 inflammasome activation by ROS and/or danger signals, including pathogen-associated molecular patterns, results in the Casp-1-dependent processing of IL-1β and IL-18 [40]. IL-1β participates in the inflammation response, activating lymphocytes, and promoting immune cell infiltration at the site of injury and inflammation. Our data show that MFP reduces the mRNA expression of IL-1β and NLRP3 inflammasomes in mice fed MFP, suggesting that MFP acts as a powerful antioxidant and cancels the ROS influence in the colonic microenvironment.

Our result of the increased expression of NLRP6 inflammasomes in mice fed MFP suggest a key role of NLRP6 in intestinal homeostasis by regulating IL-18 expression, secreting mucin, and producing anti-microbial peptides from goblet cells. The mucus overlying the epithelial cells is secreted mainly by goblet cells, and the secretion is regulated by NLRP6 inflammasomes [10]. Our results showed that goblet cells are maintained in Group 1 mice compared with Group 3 and 5 mice, suggesting that MFP promotes the formation of NLRP6 inflammasomes and preserves the goblet cell number. This might lead to protective mucus barrier in the colon, resulting in the inhibition of DSS-induced acute colitis. Moreover, NLRP6 inflammasomes can negatively regulate the NF-kB and MAPK signaling pathways to control the expression of proinflammatory cytokines [41]. NLRP6 inflammasome-deficient mice are characterized by the infiltration of inflammatory cells in the lamina propria and the exacerbation of colitis [42].

Several studies have reported that the intestinal flora plays a key role in the pathogenesis of intestinal inflammation and that intestinal microbial dysbiosis is closely associated

with the pathogenesis of DSS-induced UC [43-46]. Animal models indicate that intestinal bacteria have different proinflammatory activities in relation to the induction of colitis. For instance, *Bifidobacterium* spp., a kind of probiotic, has an inhibitory effect on DSS-induced colitis [47,48]. *Bacteroides* spp. [18,49] and *E. coli* [18] are considered as two pathogenic bacteria for colitis. *Clostridium* spp. promote the accumulation of regulatory T cells, which play a key role in mucosal immunity [50]. Some *Clostridium* species are considered as pathogens, such as *C. difficile* [51]. On the other hand, *C. butyricum*, which belongs to *C. perfringens* group, is probiotic bacteria able to reduce intestinal inflammation by improving the mucosal barrier function and modulating immune responses [52]. In this study, we determined the contents of *Bifidobacterium spp.* and *C. perfringens* group; our results indicate that those two bacterial groups were significantly increased in mice fed MFP of Group 1 compared with Group 3 and Group 5 mice that were not fed MFP (Fig 4). As these probiotic bacteria reduce mucosal inflammation by modulating cytokine production, decreasing oxidative stress, and enhancing the mucosal barrier [21,53,54], our results suggest that MFP increases immunomodulation in the colon by the increased contents of these bacteria.

In conclusion, MFP attenuates disease severity and provides mucosal protection by modifying the bacterial content, maintaining the goblet cells, and activating NLRP6 inflammasomes in DSS-induced acute colitis. MFP contains the anthocyanins, cyanidin-3-glucoside and cyanidin-3-rutinoside as functional antioxidative ingredients. These findings suggest that MFP has beneficial health effects.

Acknowledgments

We are grateful to Mr. Naoki Oonishi and Ms. Takako Fujiwara in Okayamaken Seikabutsu Hanbai Co. Ltd. for their financial support and for providing the mulberry fruit juice supplementation. We also thank Drs. Yuki Yamamoto, Koji Kimura, Takehito Tsuji, and Naoki Nishino in Okayama University for their technical equipment assistance. The authors would like to thank Enago (www.enago.jp) for the English language review.

Author Disclosure Statement

The authors declare that they have no conflict of interest regarding the publication of this article.

References

1. Lovasz BD, Golovics PA, Vegh Z, et al. New trends in inflammatory bowel disease epidemiology and disease course in Eastern Europe. Dig Liver Dis. 2013;45(4):269-276.
2. Weimers P, Burisch J. The importance of detecting irritable bowel-like symptoms in inflammatory bowel disease patients. J Crohn's Colitis. 2018;12(4):385-386.
3. Keshavarzian A, Sedghi S, Kanofsky J, et al. Excessive production of reactive oxygen metabolites by inflamed colon: analysis by chemiluminescence probe. Gastroenterology. 1992;103:177-185.
4. Jang JH, Shin HW, Lee JM, et al. An overview of pathogen recognition receptors for innate immunity in dental pulp. Mediators Inflamm. 2015 [cited 2015 Oct 20]; [12 p.]. DOI:10.1155/2015/794143
5. Vanaja SK, Rathinam VA, Fitzgerald KA. Mechanisms of inflammasome activation: recent advances and novel insights. Trends Cell Biol. 2015;25(5):308-315.
6. Błażejewski AJ, Thiemann S, Schenk A, et al. Microbiota normalization reveals that canonical Caspase-1 activation exacerbates chemically induced intestinal inflammation. Cell Rep. 2017;19(11):2319-2330.
7. Abderrazak A, Syrovets T, Couchie D, et al. NLRP3 inflammasome: from a danger signal sensor to a regulatorynode of oxidative stress and inflammatory diseases. Redox Biol. 2015;4:296-307.
8. Zaki MH, Boyd KL, Vogel P, et al. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. Immunity. 2010;32(3):379-391.

9. Elinav E, Henao-Mejia J, Flavell RA. Integrative inflammasome activity in the regulation of intestinal mucosal immune responses. Mucosal Immunol. 2013;6(1):4-13.
10. Wlodarska M, Thaiss CA, Nowarski R, et al. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. Cell. 2014;156(5):1045-1059.
11. Levy M, Shapiro H, Thaiss CA, et al. NLRP6: a multifaceted innate immune sensor. Trends Immunol. 2017;38(4):248-260.
12. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017;474(11):1823-1836.
13. Schleimer RP, Berdnikovs S. Etiology of epithelial barrier dysfunction in patients with type 2 inflammatory diseases. J Allergy Clin Immunol. 2017;139(6):1752-1761.
14. Bamola VD, Ghosh A, Kapardar RK, et al. Gut microbial diversity in health and disease: experience of healthy Indian subjects, and colon carcinoma and inflammatory bowel disease patients. Microb Ecol Health Dis. 2017 [cited 2017 May 19]; [8 p.]. DOI:10.1080/16512235.2017.1322447
15. Onderdonk AB, Cisneros RL, Bronson RT. Enhancement of experimental ulcerative colitis by immunization with *Bacteroides vulgatus.* Infect Immun. 1983;42(2):783-788.
16. Rhee KJ, Wu S, Wu X, et al. Induction of persistent colitis by a human commensal, enterotoxigenic *Bacteroides fragilis*, in wild-type C57BL/6 mice. Infect Immun. 2009;77(4):1708-1718.

17. Rath HC, Herfarth HH, Ikeda JS, et al. Normal luminal bacteria, especially *bacteroides* species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human β2 microglobulin transgenic rats. J Clin Invest. 1996;98(4):945-953.
18. Rath HC, Wilson KH, Sartor RB. Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with *Bacteroides vulgatus* or *Escherichia coli*. Infect Immun. 1999;67(6):2969-2974.
19. Lucke K, Miehlke S, Jacobs E, et al. Prevalence of *Bacteroides* and *Prevotella* spp. in ulcerative colitis. J Med Microbiol. 2006;55:617-624.
20. Zhao K, Yu L, Wang X, et al. *Clostridium butyricum* regulates visceral hypersensitivity of irritable bowel syndrome by inhibiting colonic mucous low grade inflammation through its action on NLRP6. Acta Biochim Biophys Sin (Shanghai). 2018;50(2):216-223.
21. McCarthy J, O'Mahony L, O'Callaghan L, et al. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. Gut. 2003;52(7):975-980.
22. Yoshida K, Murayama MA, Shimizu K, et al. IL-1R2 deficiency suppresses dextran sodium sulfate-induced colitis in mice via regulation of microbiota. Biochem Biophys Res Commun. 2018;496(3):934-940.
23. Cosnes, J. Can we modulate the clinical course of inflammatory bowel diseases by our current treatment strategies? Dig Dis. 2009;27(4):516-521.
24. Boussenna A, Cholet J, Goncalves-Mendes N, et al. Polyphenol-rich grape pomace extracts protect against dextran sulfate sodium-induced colitis in rats. J Sci Food Agric. 2016;96(4):1260-1268.

25. Ercisli S, Orhan E. Some physico-chemical characteristics of black mulberry (*Morus nigra L.*) genotypes from Northeast Anatolia region of Turkey. Sci Hortic. 2008;116(1):41-46.
26. Rahman HS, Muhammad KS, Sattar FA, et al. The effectiveness of super ovulation and multiple pregnancies in sprague dawley rat using *Morus alba Linn.* fruit. Int J Med Res Heal Sci. 2018;7(1):17-26.
27. Kim AJ, Park S. Mulberry extract supplements smeliorate the inflammation-related hematological parameters in carrageenan-induced arthritic rats. J Med Food. 2006;9(3):431-435.
28. Li L, Wang L, Wu Z, et al. Anthocyanin-rich fractions from red raspberries attenuate inflammation in both RAW264.7 macrophages and a mouse model of colitis. Sci Rep. 2014 [cited 2014 Aug 29]; [11 p.]. DOI: 10.1038/srep06234
29. Dieleman LA, Palmen MJ, Akol H, et al. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. Clin Exp Immunol. 1998;114(3):385-391.
30. Rinttilä T, Kassinen A, Malinen E, et al. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol. 2004;97(6):1166-1177.
31. Martini E, Krug SM, Siegmund B, et al. Mend your fences: the epithelial barrier and its relationship with mucosal immunity in inflammatory bowel disease. Cell Mol Gastroenterol Hepatol. 2017;4(1):33-46.
32. Stevceva L, Pavli P, Husband AJ, et al. The inflammatory infiltrate in the acute stage of the dextran sulphate sodium induced colitis: B cell response differs depending on the percentage of DSS used to induce it. BMC Clin Pathol. 2001 [cited 2001 Sep 10]; [11 p.]. DOI: 10.1186/1472-6890-1-3

33. Wu LH, Xu ZL, Dong D, et al. Protective effect of anthocyanins extract from blueberry on TNBS-induced IBD model of mice. Evid Based Complement Alternat Med. 2011 [cited 2011 Apr 14]; [8 p.]. DOI:10.1093/ecam/neq040
34. Jin YS, Sa JH, Shim TH, et al. Hepatoprotective and antioxidant effects of *Morus bombycis* Koidzumi on CCl4-induced liver damage. Biochem Biophys Res Commun. 2005;329(3):991-995.
35. Yang X, Yang L, Zheng H. Hypolipidemic and antioxidant effects of mulberry (*Morus alba L.*) fruit in hyperlipidaemia rats. Food Chem Toxicol. 2010;48(8-9):2374-2379.
36. Andallu B, Suryakantham V, Lakshmi Srikanthi B, et al. Effect of mulberry (*Morus indica L.*) therapy on plasma and erythrocyte membrane lipids in patients with type 2 diabetes. Clin Chim Acta. 2001;314(1-2):47-53.
37. Naowaboot J, Pannangpetch P, Kukongviriyapan V, et al. Antihyperglycemic, antioxidant and antiglycation activities of mulberry leaf extract in streptozotocin-induced chronic diabetic rats. Plant Foods Hum Nutr. 2009;64(2):116-121.
38. Enkhmaa B, Shiwaku K, Katsube T, et al. Mulberry (*Morus alba L.*) leaves and their major flavonol quercetin 3-(6-malonylglucoside) attenuate atherosclerotic lesion development in LDL receptor-deficient mice. J Nutr. 2005;135(4):729-734.
39. Sugimoto M, Arai H, Tamura Y, et al. Mulberry leaf ameliorates the expression profile of adipocytokines by inhibiting oxidative stress in white adipose tissue in db/db mice. Atherosclerosis. 2009;204(2):388-394.
40. de Zoete MR, Palm NW, Zhu S, et al. Inflammasomes. Cold Spring Harb Perspect Biol. 2014 [cited 2014 Oct 16]; [22 p.]. DOI:10.1101/cshperspect.a016287

41. Anand PK, Malireddi RK, Lukens JR, et al. NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. Nature. 2012;488(7411):389-393.
42. Elinav E, Strowig T, Kau AL, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell. 2011;145(5):745-757.
43. Cao X. Intestinal inflammation induced by oral bacteria. Science. 2017;358(6361):308-309.
44. Sasaki M, Klapproth JM. The role of bacteria in the pathogenesis of ulcerative colitis. J Signal Transduct. 2012 [cited 2012 Apr 24]; [6 p.]. DOI:10.1155/2012/704953
45. Knights D, Lassen KG, Xavier RJ. Advances in inflammatory bowel disease pathogenesis: linking host genetics and the microbiome. Gut. 2013;62(10):1505-1510.
46. Goto Y, Kurashima Y, Kiyono H. The gut microbiota and inflammatory bowel disease. Curr Opin Rheumatol. 2015;27(4):388-396.
47. Venturi A, Gionchetti P, Rizzello F, et al. Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. Aliment Pharmacol Ther. 1999;13(8):1103-1108.
48. Nanda Kumar NS, Balamurugan R, Jayakanthan K, et al. Probiotic administration alters the gut flora and attenuates colitis in mice administered dextran sodium sulfate. J Gastroenterol Hepatol. 2008;23(12):1834-1839.
49. Bloom SM, Bijanki VN, Nava GM, et al. Commensal *Bacteroides* species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. Cell Host Microbe. 2011;9(5):390-403.

50. Atarashi K, Tanoue T, Shima T, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. Science. 2011;331(6015):337-341.
51. Sun YY, Li M, Li YY, et al. The effect of *Clostridium butyricum* on symptoms and fecal microbiota in diarrhea-dominant irritable bowel syndrome: a randomized, double-blind, placebo-controlled trial. Sci Rep. 2018 [cited 2018 Feb 14]; [11 p.]. DOI: 10.1038/s41598-018-21241-z
52. Banaszkiewicz A, Kądzielska J, Gawrońska A, et al. Enterotoxigenic *Clostridium perfringens* infection and pediatric patients with inflammatory bowel disease. J Crohns Colitis. 2014;8(4):276-281.
53. Amaretti A, di Nunzio M, Pompei A, et al. Antioxidant properties of potentially probiotic bacteria: *in vitro* and *in vivo* activities. Appl Microbiol Biotechnol. 2013;97(2):809-817.
54. Madsen KL, Doyle JS, Jewell LD, et al. *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology. 1999;116(5):1107-1114.

Tables with captions

Table 1. The grouping of mice.

Table 2. The scoring system for Disease activity index.

Table 3. The scoring system for Histological damages.

Table 4. The Primer sets for cytokine and NLRPs.

Figure legends

Figure 1. MFP significantly attenuated the clinical symptoms of colitis.

A Mulberry diet inhibits colon inflammation produced in DSS-induced colitis. During the 5% DSS-induced week, (A) daily weights were measured (n = 3/group) and calculated as a percentage of body weight change from the first day of the week. At the end of day 7, all the mice were sacrificed, and their colonic tissues were collected. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) The length of the colonic tissues was measured. (C) DAI scores were measured according to the fecal condition and body weight loss. All data are represented as the mean ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software. Different letters (a, b, and c) indicate significant differences between groups at $p < 0.05$.

Figure 2. Histopathologic results shown by HE stains.

At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected. (A) HE-stained specimens observed under light microscopy. The arrowheads indicate the infiltration of inflammatory cells. The arrows indicate the goblet cells. (B) The microscopic scores are indicated. The microscopic score was calculated according to the scoring system. (C) The number of goblet cells was measured. All data are represented as the mean ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software. MD Mulberry diet; ND Normal diet; w week; DSS dextran sodium sulfate; D day; G Group. Different letters (a, b, and c) indicate significant differences between groups at $p < 0.05$.

Figure 3. mRNA expression levels of cytokines and NLRPs in colonic tissues.

At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected. mRNA expression levels of cytokines (IL-1β and TNF-α) and NLRPs (NLRP3, ASC, and NLRP6) in colonic tissues from the mice with/without MFP and DSS. All data are represented as the mean ± SE of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software.

Figure 4. qPCR detection of total bacteria.

The fecal samples for qPCR detection of bacteria were collected at the end of experiments. Fecal DNA samples from mice in all the 6 groups were extracted, and qRT-PCR amplification and detection were performed. *Bacteroides* spp., *Bifidobacterium* spp., and *C. perfringens* subgroup contents are presented as the bacterial copy number in fecal microbiota. Different letters (a, b, c, d, and e) indicate significant differences between groups at $p < 0.05$. All data are represented as the mean ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software.